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HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes

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The human papillomavirus types (HPVs) most often associated with cancer of the cervix, such as HPV16, have been reported previously to immortalize normal human foreskin keratinocytes *in vitro*, while the types that are primarily associated with benign cervical lesions failed to do so. In this study we have determined the HPV16 genes that are responsible for the immortalizing activity of the viral genome. Transfection with a plasmid in which E6 and E7 were the only intact open reading frames (ORFs) induced an indefinite life-span in the keratinocytes with an efficiency similar to that of the entire early region of the viral DNA. Mutants in the E6E7 clone with inactivating lesions in E6 or E7 failed to induce immortalization. When transfected alone, E7 could induce hyperproliferation, but these cells eventually senesced. By itself, E6 exhibited no activity. Co-transfection of a plasmid with an intact E6 ORF and a second plasmid with an intact E7 ORF generated keratinocyte lines with indefinite growth potential. The E6 and E7 proteins were detected in the lines induced by the E6E7 DNA and by co-transfection of the E6 and E7 plasmids. Therefore, we conclude that HPV16 E6 and E7 cooperate to immortalize human keratinocytes *in vitro*. Changes in cellular gene expression are probably also required for immortalization since all of the keratinocyte lines examined were aneuploid. Serum and calcium resistant sublines were isolated from the E6E7 induced lines, indicating that other HPV genes do not play an obligatory role in the generation of resistance to differentiation. Since HPV immortalization of keratinocytes *in vitro* is likely to correlate with an important step in tumor progression *in vivo*, our results provide strong experimental support for the hypothesis that the observed maintenance and expression of E6 and E7 in human cervical carcinomas has pathologic significance.

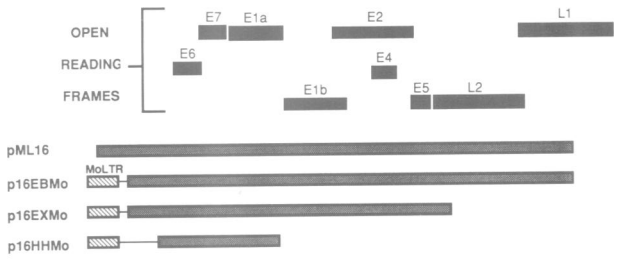
Key words: E6/E7/HPV16/immortalization/keratinocytes

Introduction

Human papillomaviruses (HPVs) are epitheliotropic viruses that induce epithelial hyperproliferation, including cutaneous warts and condylomas in cervical and vaginal epithelia (for reviews see Broker and Botchan, 1986; McCance, 1986). DNA from several genetically distinguishable types of HPVs (6, 11, 16, 18, 31, 33 and 35) has been identified in genital lesions and a subset of this group is frequently identified in genital cancers (16, 18, 31, 33 and 35). More than 90% of cervical carcinomas contain HPV DNA, suggesting that the HPV genome may play a central role in the development of these tumors. Non-viral factors are likely to be required in addition, since benign infections progress to carcinomas relatively infrequently and usually after a long latency (reviewed in Pfister, 1987; zur Hausen and Scheider, 1987).

Certain HPV genomes have been shown to have *in vitro* activity in their natural host cells. When transfected onto normal human foreskin keratinocytes or human cervical epithelial cells, these genomes were able to indefinitely extend the proliferative capacity of the cells (Durst *et al.*, 1987; Pirisi *et al.*, 1987; Kaur and McDougall, 1988; Woodworth *et al.*, 1988). This activity is commonly referred to as 'immortalization'. It is noteworthy that the HPV types closely associated with cervical malignancies (HPV16, 18, 31 and 33) were able to immortalize these cells, while those types normally associated only with benign cervical lesions (HPV6 and 11) were not (Schlegel *et al.*, 1988; Pecoraro *et al.*, 1989; Woodworth *et al.*, 1989). The close correlation between *in vitro* immortalizing activity and malignant potential suggests that these two properties may be manifestations, at least in part, of the same viral activities.

In human genital carcinomas and carcinoma-derived cell lines, the HPV DNA is usually integrated into the host genome (Durst *et al.*, 1983; Boshart *et al.*, 1984; Pater and Pater, 1985; Yee *et al.*, 1985). This integration event



CONSTRUCT	READING FRAMES	POSITIVES/ TRANSFECTIONS
Control	none	0/2
pML16	all	6/8
p16EBMo	all	5/5
p16EXMo	all	3/3
p16HHMo	E6,E7	4/5

Fig. 1. HPV16 E6-E7 region extends the life-span of human keratinocytes. The ORFs of HPV16 included in the clones are diagrammed in the map above. The E1 ORF is split between two reading frames because the full-length HPV16 clone used in generating the constructs is known to have a frame-shift at this location (Durst *et al.*, 1983). Transfections were scored as positives if the cultures continued to proliferate for 2 months (at least three passages) beyond the senescence of the pSV2Neo transfected controls. The numerator indicates the number of positive cultures, the denominator the number of cultures transfected. Results represent the combined data of three separate experiments.

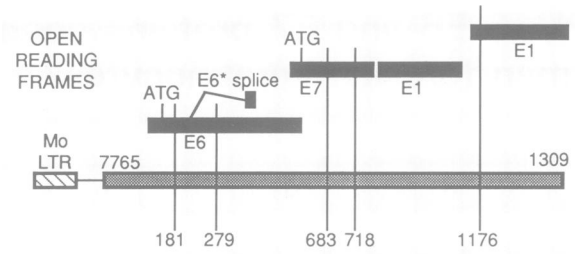
generally occurs in the E1/E2 region of the viral genome, causing a disruption and/or separation of the 3′ early open reading frames (ORFs) from the viral upstream regulatory region (URR) (Matsukura *et al.*, 1986; Baker *et al.*, 1987). Analyses of HPV transcripts and proteins in the carcinomas and derived cell lines indicate that the 5′ early ORFs, E6 and E7, are selectively retained and expressed (Schneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986). Continued expression of these genes appears to be required for proliferation of a carcinoma-derived line (von Knebel Doeberitz *et al.*, 1988). These results suggest that E6 and E7 may contribute to the development or maintenance of the carcinomas, although other HPV genes may be important during the initial stages of tumor development.

Because of the close correlation between the ability of HPVs to immortalize human keratinocytes *in vitro* and their apparent oncogenic potential, we have begun an investigation into the mechanism of HPV-induced immortalization of foreskin keratinocytes. As a first step we have sought to determine the HPV genes responsible for this activity. In this report we document that HPV16 E6 and E7 cooperate in the immortalization process. E7 alone can induce hyper-proliferation and limited extension of culture life-span but E6 is also required to extend the life-span indefinitely.

Results

Initially, the ability of the full-length HPV16 genome and of subgenomic fragments to increase the proliferative capacity of neonatal human foreskin keratinocytes was compared (Figure 1). The full-length HPV16 DNA was under the promotional control of its own URR, while the subgenomic constructions were promoted by a murine retroviral LTR. In three separate experiments, up to eight individual transfections per construction were analyzed for extended life-span. Transfected cells were grown under non-differentiating conditions in KGM and subcultured as the cells approached confluence. Cells transfected with control DNAs senesced one or two passages after transfection. All of the HPV16-expressing clones gave rise to proliferating lines with similar efficiencies. Cultures that continued to proliferate for 2 months after senescense of the controls (at least three passages) were scored as positive in the assay. The construction p16HHMo, which contained only the E6 and E7 ORFs and a small part of the E1 ORF, was fully active. One of the lines generated using this construct has been in continuous culture for 9 months and is in its 21st passage post-transfection. Given the length of time these cells have been in culture and the number of passages since the control cells senesced, our expectations are that this, and other similar lines, will continue to grow indefinitely. It was not possible to assay the number of individual events per transfection because, under the non-selective conditions used, the cells required subculturing at least once before the controls senesced. However, because of the rapidity with which proliferating cells dominated some of the transfected cultures (particularly those transfected with the LTR-activated constructions), we suspect that many of the lines were, at least initially, polyclonal.

To determine if only E6, E7 or both genes were responsible for the induction of an indefinite life-span in the keratinocytes, linkers with translation termination sequences in all three reading frames were introduced into p16HNMo,



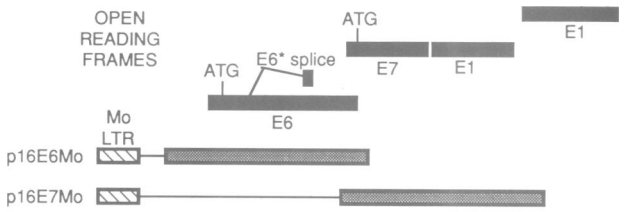
CONSTRUCT	INTACT ORFs E6	E6*	E7	POSITIVES/ TRANSFECTIONS
p16HHMo	+	+	+	4/5
p16-1176Mo	+	+	+	3/8
p16-181Mo	-	-	+	0/8
p16-279Mo	-	+	+	0/6
p16-683Mo	+	+	-	0/2
p16-718Mo	+	+	-	0/6
p16-181/718Mo	-	-	-	0/5
p16-279/718Mo	-	+	-	0/5
p16-181Mo + p16-718Mo	+	+	+	4/6
p16-279Mo + p16-718Mo	+	+	+	2/6
p16-181Mo + p16-279/718Mo	-	+	+	0/3

Fig. 2. Both E6 and E7 ORFs are required for extension of keratinocyte life-span. The nucleotides at the 5′ and 3′ ends of the HPV16 sequence included in the construct p16HNMo are indicated above the stippled bar and the nucleotides adjacent to the inserted termination linker are indicated below the bar. p16-181/718Mo and p16-279/718Mo each contained two mutations at the locations named. Results were analyzed as described in the legend to the Figure 1.

a construct identical to p16HHMo except that it lacked 643 bp at the 3′ terminus of the latter clone (Vousden *et al.*, 1988). Figure 2 shows the locations of the various mutants tested in relation to the ORFs present in the p16HNMo clone. Since all the E6 and E7 mutants were defective, the results indicate that, when expressed from a common promoter, both reading frames were required for extending the life-span of the keratinocytes.

The only mutant which produced proliferating lines by itself was p16-1176Mo, which interrupted the truncated E1 ORF in p16HNMo but retained the E6 and E7 as intact ORFs (Figure 2). Interruptions in E6 which eliminated both full-length E6 and E6* (a potential truncated E6 protein translated from the spliced message) (at nt 181) or only E6 (at nt 279) were inactivating, as were two different interruptions of E7 (at nt 683 or nt 718). The negative result with the mutant at nt 279 suggests that an E6* product cannot functionally substitute for E6 in the immortalization of keratinocytes. This conclusion is tentative because we have not been able to detect an E6* protein in cells containing this (or any other) HPV16 clone with our E6 antiserum. As expected, the double mutants that interrupted both E6 and E7 (181/718 and 279/718, which could potentially express E6*) were also negative.

We were concerned that the two E6 mutants might be negative in the assay because they interfered with the expression of E7 in the keratinocytes. To rule out this possibility, we determined whether the E6 mutants expressed a functional E7 product (and the E7 mutants expressed a functional E6 product), by co-transfecting pairs of individual



CONSTRUCT	POSITIVES/ TRANSFECTIONS
p16E6Mo	0/6
p16E7Mo	5/6*
p16E6Mo + p16E7Mo	3/6

Fig. 3. E7 alone can induce transient proliferation of keratinocytes. Results were analyzed as described in the legend for Figure 1. The asterisk indicates that these cultures grew more slowly than the positive controls and eventually senesced.

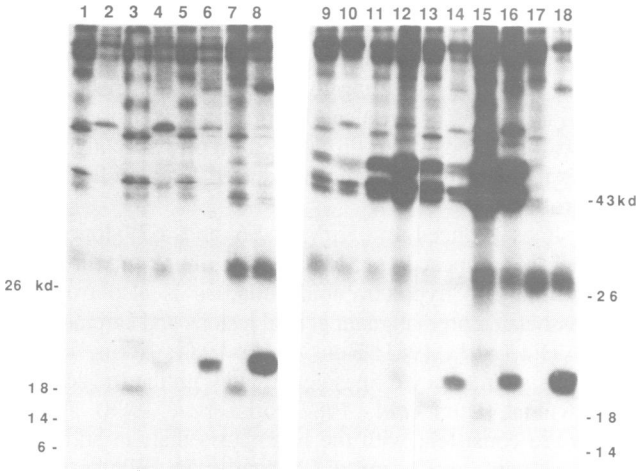


Fig. 4. Immunoprecipitation of HPV16 E6 and E7 proteins from keratinocyte lines with extended life-spans. Cell lines were metabolically labeled and immunoprecipitated as described in Materials and methods. Precipitates were resolved on two separate SDS−15% polyacrylamide gels (**lanes 1−8** and **9−18**). Positions of ^{14}C-labeled mol. wt standards are indicated. Extracts precipitated with antiserum to E6 are in odd-numbered lanes, those with antiserum to E7 are in even-numbered lanes. Extracts were from the following cell lines: **lanes 1, 2, 9** and **10**, a negative control line immortalized with an SV40 LT expression vector (line 80, seventh passage after transfection); **lanes 7, 8, 17** and **18**, an HPV16 positive control, CaSki cell line; **lanes 3** and **4**, p16HHMo (line 9, seventh passage); **lanes 5** and **6**; p16E7Mo (culture 63, fifth passage); **lanes 11** and **12**, pML16 (line 6A, eighth passage); **lanes 13** and **14**, co-transfection with p16-181Mo and p16-718Mo (line 32, fifth passage); **lanes 15** and **16**, co-transfection with p16E6Mo and p16E7Mo (line 68, seventh passage).

mutants. When the single mutants were co-transfected in pairs which would provide uninterrupted E6 and E7 ORFs on separate plasmids (181 and 718; 279 and 718), the ability of these constructions to extend the life-span of the keratinocytes was restored (Figure 2). To examine the possibility that this result might be due to recombination between the two mutants in these co-transfections, the combination of 181 and 279/718 was also tested. These two mutants cannot complement each other in *trans* to provide a wild-type E6, but they could potentially recombine with each other to generate an intact E6 ORF. Since this combination was negative in the assay, we conclude that the E6 and E7 mutants primarily complement each other in *trans* by expressing their respective gene products.

The above experiments demonstrate that in the construction with the full E6−E7 region behind a common promoter, both E6 and E7 are required to induce an extended life-span in the keratinocytes. It is likely that in these constructions, as in the HPV16-containing carcinoma-derived cell lines, E7 is translated from a spliced form of the E6 message and, therefore, that the levels of the two proteins are determined, at least in part, by the frequency of message splicing (Smotkin *et al.*, 1989). In an attempt to maximize the expression of each gene individually, we constructed expression vectors with the Moloney LTR immediately adjacent to either E6 or E7 (p16E6Mo or p16E7Mo respectively), removing the splice donor−acceptor pair from the E7 expression vector. When transfected alone, p16E6Mo had no activity (Figure 3). This construction expresses a functional E6 product because it was able to complement p16E7Mo in generating vigorously growing keratinocyte cultures with indefinite life-spans (currently 8 months of continous culture) that are comparable to the cultures induced by p16HHMo and these lines express E6 protein (see below). In contrast to p16E6Mo, p16E7Mo alone reproducibly increased the proliferative capacity of the transfected keratinocytes. However, these cultures grew more slowly than those transfected with both E6 and E7, requiring passage approximately half as frequently, and eventually senesced four to six passages after transfection. Prior to senescence, these cultures contained an amount of E7 protein that was comparable to that found in lines that were co-transfected with E6 (see below).

To demonstrate the E6 and E7 proteins expressed from the biologically active constructs in the induced keratinocyte lines, the proliferating keratinocytes were labeled with [^{35}S]cysteine and the cell extracts were immunoprecipitated with previously characterized antisera against E6 and against E7. The CaSki cervical carcinoma-derived cell line, which we had previously shown to express both E6 and E7 proteins (Androphy *et al.*, 1987), was used as a positive control; an SV40 LT-induced human keratinocyte line generated in parallel with the HPV16-induced lines was used as a negative control. Representative results are shown in Figure 4. E7 was detected in all lines with extended life-span regardless of the HPV16 construction used, including a culture transfected with the E7 vector alone that senesced after an additional subculture. As is true of HPV-positive human cell lines derived from cervical carcinomas, E6 was generally more difficult to detect. It was usually apparent in the lines that were transfected with the LTR-activated constructions but was often undetectable in lines induced by the full-length genome. The relative amounts of the two proteins varied among the 13 lines tested and there was no consistent correlation between the ratios of the two proteins and any obvious growth characteristic of the lines.

Many lines immortalized by HPV DNA have been reported to be resistant to high calcium- or serum-induced terminal differentiation (Kaur *et al.*, 1988; Schlegel *et al.*, 1988; Woodworth *et al.*, 1988). To determine if cells expressing only E6 and E7 displayed this property, representative lines induced by the various HPV16 constructions were tested for resistance to serum- and calcium-induced

Table I. Number of chromosomes per cell in immortalized cell lines

Line	Construction	Chromosome number
NHK	none	43 44 46 46 46 46 47 47 48
1B2	HPV16pML	78 78 79 82 83 84 84 85 85 86 86 86 87
9	p16HHMo	42 42 43 43 43 43 44 44 45 45 82 82 85 85 87 165
11	p16HHMo	42 43 43 43 43 44 45 76 78 78 79 82 83
32	p16-181Mo + p16-718Mo	41 42 42 43 44 77 79 81 83 85 88 91 156
68	p16E6Mo + p16E7Mo	81 85 85 85 86 86 87 87 87 87 90 93 96
80	SV40 LT	42 72 76 77 79 80 82 82 83 84 84 84 88 88 89 91 92 93

differentiation by exposing the proliferating keratinocytes to DMEM containing 10% fetal calf serum for 2 weeks, followed by subculturing in a 3:1 mixture of KGM:DMEM with 10% serum (Schlegel *et al.*, 1988). Under these conditions, normal human keratinocytes differentiate and die. Most of our lines generated by the LTR-activated E6E7 fragment, by the co-transfection of the E6 and E7 mutants or by the co-transfection of the E6 and E7 only expression vectors, contained at least a minority of cells that were capable of surviving this treatment. With continuous subculturing in 3:1 medium, those DMEM selected sublines have shown no indication of senescence. In fact, the sublines generally grow more rapidly in 3:1 medium than the parental lines grow in KGM (data not shown). From these results we conclude that other HPV16 genes do not play an obligatory role in the emergence of keratinocytes that are resistant to differentiation.

Since human cervical carcinomas and keratinocyte lines established after transfection of the full-length HPV16 genome are aneuploid (Fu *et al.*, 1981; Reid *et al.*, 1984; Durst *et al.*, 1987; Pirisi *et al.*, 1988), we analyzed representative lines generated by our constructs to determine if they also contained aberrant numbers of chromosomes. The number of chromosomes in colcemid-arrested metaphases were counted after staining with Giemsa dye (Table I). The untransfected normal keratinocytes were diploid, as expected. The lines induced by the full-length HPV16 (1B2) and the SV40 LT (80) controls were, in agreement with previous reports, aneuploid. The four lines transfected with only E6 and E7 contained either hypotetraploid or a mixture of hypodiploid and hypotetraploid cells. All of the lines contained cells with hypo-octoploid and greater numbers of chromosomes. However, except in the two instances indicated in Table I (lines 9 and 32), accurate counts of these metaphases were not possible.

Discussion

This study demonstrates that HPV16 E6 and E7 proteins cooperate to induce long-term extension of human genital keratinocyte life-span in culture. This is the first report to identify the specific HPV-encoded proteins responsible for this activity. E7 alone could stimulate proliferation of the keratinocytes beyond the senescence of the controls, but these cultures failed to become immortalized. Since human keratinocyte immortalization has been shown to occur only under the influence of HPV types associated with malignant lesions, this observation suggests that the maintenance of both E6 and E7 ORFs in cancerous tissues has functional significance, and that this assay monitors an important step in the carcinogenic process.

We chose to assay for the induction of an indefinite life-span in the keratinocytes under the least stringent conditions possible and so grew the cells in medium which did not stimulate the terminal differentiation of normal cells (low calcium, no serum). We were concerned that if, as in some studies, both resistance to differentiation and extended proliferation were assayed simultaneously, we might be unable to distinguish the genes necessary and sufficient to individually induce either of these two phenotypes. Most of our lines contained a subset of cells that were able to proliferate in the presence of serum and a higher calcium concentration. However, some lines did not yield serum-resistant sublines, indicating that differentiation resistance is not a necessary prerequisite to life-span extension. This suggests that resistance to differentiation probably involves changes in the cell beyond those required for stimulation of proliferation.

Our lines induced by HPV16 E6 and E7 alone are aneuploid, composed predominantly of a mixture of hypodiploid and hypotetraploid cells, as is also true of HPV-associated premalignant genital lesions and carcinomas *in vivo* (Fu *et al.*, 1981; Reid *et al.*, 1984; Atkin, 1986) and human keratinocyte lines generated with the full-length HPV16 clone (Durst *et al.*, 1987; Pirisi *et al.*, 1988). These results are consistent with the hypothesis that, in addition to the activity of E6 and E7, changes in cellular gene expression are required for the immortalization of human keratinocytes. Chromosome rearrangements may also contribute to the development of serum-resistant cells in the cultures. If papillomavirus gene products are involved in the generation of aneuploidy, then our results would implicate the E6 and/or E7 proteins in this function. However, the direct involvement of viral proteins is not proven by this analysis, since we cannot rule out the possibility that the observed chromosomal aberrations may have been induced by the introduction of exogenous DNA.

The finding that E6 and E7 cooperate to extend indefinitely the life-span of human keratinocytes is particularly interesting since neither gene can be easily categorized according to the 'transformation' versus 'immortalization' scheme commonly used to classify viral and cellular oncogenes. E7 is capable of transforming immortal rodent fibroblast cell lines to anchorage independence (Kanda *et al.*, 1988; Phelps *et al.*, 1988; Vousden *et al.*, 1988; Tanaka *et al.*, 1989) and stimulating DNA synthesis in those cells (Sato *et al.*, 1989a). It can also provide the 'immortalization' function in cooperating with the oncogenes *ras* or *fos* in malignant transformation of primary rodent cells (Crook *et al.*, 1988) and in functionally substituting for a temperature sensitive SV40 LT in a cell line that is LT dependent for growth (Vousden and Jat, 1989). This phenotype appears to be dependent upon the continuous expression of E7 (Crook *et al.*, 1989).

HPV16 and 18 E6 genes do not cooperate with *ras* to immortalize primary rodent fibroblasts (Storey *et al.*, 1988; Bedell *et al.*, 1989). A non-tumorigenic rodent cell line transfected with an HPV16 E6 expression vector, whilst showing only slight anchorage independence, was capable of forming tumors (Yutsudo *et al.*, 1988). HPV16 E6 has also been reported to cooperate with E7 to induce hyperproliferation of primary human fibroblasts but these cells were not immortalized (Watanabe *et al.*, 1989).

The corresponding bovine papillomavirus (BPV) E6 can transform the mouse C127 line to a fully tumorigenic phenotype but is inactive in the commonly assayed NIH3T3 line (Schiller *et al.*, 1984). BPV E6 cannot independently induce proliferation of rat embryo fibroblasts (REF), but it can immortalize colonies of REFs stimulated to proliferate with BPV E5 or E2 (Cerni *et al.*, 1989). The activity of BPV E6 in the REF assay therefore resembles the activity of HPV16 E6 in the human keratinocyte assay reported here, suggesting that the two E6s may perform the same function by similar mechanisms in the two assays.

The E6 and E7 proteins appear to be evolutionarily related and may have arisen by duplication and divergence of a primordial gene containing a Cys-x-x-Cys repeat (Cole and Danos, 1987). These repeats have been implicated in the ability of E6 and E7 to bind zinc (Barbosa *et al.*, 1989). However, the two proteins have been reported to differ in subcellular localization. HPV16 E7 is a phosphoprotein that has been localized to the cytoplasm by immunoprecipitation and to the nucleus by immunofluorescence (Smotkin and Wettstein, 1987; Sato *et al.*, 1989b), while HPV18 and HPV16 E6 have been localized to the nuclear matrix and non-nuclear membranes by immunoprecipitation (Grossman *et al.*, 1989; our unpublished observation).

The elucidation of the mechanisms through which E6 and E7 exert their biological effects has only recently begun. A region in the genital HPV E7 proteins has homology with one in SV40 LT and adenovirus E1a protein and like E1a, E7 is capable of *trans*-activating the adenovirus E2 promoter (Phelps *et al.*, 1988). This segment of E7, which is required for *trans*-activation and also fibroblast transformation (Edmonds and Vousden, 1989), includes a sequence that may be involved in binding the retinoblastoma-associated tumor suppressor gene product RB (Dyson *et al.*, 1989). Experiments are in progress to determine if this region, and by implication RB binding, is critical for extending the life-span of the human keratinocytes.

HPV18 E6 has been reported to activate papillomavirus transcription, possibly by an indirect mechanism (Gius *et al.*, 1988). This activation appears to be mediated through a 5′ segment of the URR. It is unlikely that, in our assay, E6 cooperates with E7 by activating E7 expression, since it cooperates with an E7 expression vector that is under the control of a heterologous promoter lacking URR sequences. Furthermore, cells which senesced because they were transfected with E7 alone contained levels of E7 protein that were comparable to those in the immortalized lines. However, it is possible that E6 acts by regulating the expression of cellular genes.

Materials and methods

Cell culture and transfection

Cultures of normal human keratinocytes derived from neonatal foreskins were purchased from the Clonetics Corporation (San Diego, CA) and were maintained continuously in modified MCDB153 (KDM, Clonetics, San Diego, CA) supplemeneted with 4 ml/l bovine pituitary extract (Hammond Cell Tech, Alameda, CA) to produce KGM. Cultures were fed 2–3 times per week, subcultured weekly and transfected at 11 population doublings after their arrival in our laboratory, a few doublings before they normally senesced (Schlegel *et al.*, 1988).

Cells were transfected with undigested plasmid DNA using polybrene and a DMSO shock, as described by Farr *et al.* (1987). Briefly, 5×10^5 cells were plated per 60 mm dish the day before transfecting. Cells were exposed to 8 μg of plasmid DNA in KGM containing 25 μg/ml polybrene for 6 h at 37°C. A 2 min 18% DMSO shock followed, then the cells were washed with saline and fed fresh medium. After 3 days, the plates were trypsinized and the cells replated on two 100 mm dishes. These cultures were fed with KGM and passaged 1:4 to 1:8 as they approached confluence.

Plasmids

pML16 contains the complete HPV16 genome isolated by Durst *et al.* (1983) cloned at its *Bam*HI site into the pBR322 derivative pML2 (Lusky and Botchan, 1981). The previously described p16EBMo, p16EXMo, p16HHMo and p16HNMo contain subgenomic HPV16 fragments cloned in pML2 and are under promotional control of the Moloney LTR (Vousden *et al.*, 1988). Premature termination mutations in reading frames E6, E7 and E1 were produced in subgenomic clone p16HNMo by insertion of an oligonucleotide linker with termination codons in all three reading frames at locations indicated in Figure 2 (Vousden *et al.*, 1988). Mutants were designated according to the nucleotide at the 5′ end of the insertion site. Constructs p16E6Mo and p16E7Mo, expressing E6 and E7 indvidually under control of the Moloney LTR and without alternative splicing, were cloned in pUC19 as previously described (Vousden and Jat, 1989).

Immunoprecipitations

Immunoprecipitations were performed as previously described (Androphy *et al.*, 1987). Cells were labeled for 1 h with 1 mCi [^{35}S]cysteine. After preclearing of the lysates with protein A–Sepharose coated with rabbit IgG, 10^7 c.p.m. were precipitated with protein A–Sepharose complexed with affinity-purified polyclonal rabbit anti-HPV16 E6 serum (Androphy *et al.*, 1987) or polyclonal rabbit anti-HPV16 E7 serum (Firzlaff *et al.*, 1987). After boiling in SDS and β-mercaptoethanol, the immunoprecipitates were analyzed by SDS–PAGE and autoradiography.

Cytogenetic analysis

Subconfluent cultures were treated overnight with 0.04 μg/ml colcemid in KGM. Cells were harvested with trypsin/EDTA, pelleted, resuspended in 75 mM KCl for 20 min and fixed by washing four times in methanol:acetic acid (3:1) according to the method of Yunis and Chandler (1977). The fixed cells were spread on glass slides that had either been precooled to −20°C or wetted in 0.01% NP40 at 4°C. The slides were air dried and then stained with Giemsa stain (Gibco, Grand Island, NY).

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